Molecular diagnostics by microelectronic microchips

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ABSTRACT

Molecular diagnostics are being revolutionized by the development of highly advanced technologies for DNA and RNA testing. One of the most important challenges is the integration of microelectronics to microchip-based nucleic acid technologies. The specific characteristics of these microsystems make the miniaturization and automation of any step of a molecular diagnostic procedure possible. This review describes the application of microelectronics to all the processes involved in a genetic test, particularly to sample preparation, DNA amplification and sequence variation detection.

INTRODUCTION

Molecular diagnostics represent a discipline which combines laboratory medicine with the knowledge and technology of molecular genetics. Its aim is to provide a sensitive alternative to protein-based current methodologies by developing DNA/RNA-based analytical methods for monitoring human pathologies.

The large amount of DNA sequence information, which has become available due to the completion of the Human Genome Project, has permitted the discovery and identification of an increasing number of genetic variations leading to the rapid, ongoing molecular characterization of a large number of monogenic diseases. The identification of genes involved in multifactorial diseases is more complex and goes on more slowly, and is mainly based on some large international projects targeted to the search and identification of sequence variations. The large collection of mapped single nucleotide polymorphisms (SNP) would provide a powerful tool for human genetic studies. An average of one SNP every 1000-2000 base pairs has been estimated in the human genome and the need for high throughput molecular assays for their detection is growing dramatically (1-3).

One of the great challenges of modern molecular biology is the integration of this new genetic information into diagnostic procedures. As a result, molecular diagnosis should be implemented with rapid and cost-effective methods for efficient identification and treatment of disease-related phenotypes. Sensitive and simple to perform, innovative genotyping methods are urgently needed for gaining full access to inter-individual genetic variations.

A gap exists between the continuously increasing genetic information and the methods presently available in diagnostic laboratories. These techniques are cumbersome, time consuming, demand a lot of practical work and are not suitable for large population screenings. To overcome such limitations, it is mandatory to develop new molecular approaches, which combine a high diagnostic reliability with high-throughput performance at low cost.

Possibly, the most promising DNA diagnostic tool is the so called "DNA-chip", a small device that holds a regular array of DNA molecules. The fundamental principle of most DNA-chips is the highly selective nature of DNA double helix hybridization. Perfectly complementary sequences are selectively identified due to their ability to hybridize with a greater efficiency than imperfectly matched ones (4).

Microchip-based nucleic acid technology has potential for sample detection in integrated systems and permits the rapid analysis of genetic information by miniaturization of many cumbersome techniques, thus allowing assay development for the rapid detection of many SNPs in a large population sample (5-10).

Theoretically, the strategy of choice may be the adaptation and use of the microarray technology, coupled with complete automation and optimization of all preanalytical procedures for sample preparation. At present, one of the most promising approach in this field is the integration of microarray technology with microelectronics based on the use of several different research disciplines including physics, chemistry and engineering (11-14). Microelectronics exploit the silicon processing industry to integrate mechanical elements, sensors, actuators and electronics on a common silicon substrate through microfabrication technology, making possible the realization of complete “systems-on-a-chip”. Miniaturization of mechanical systems allows to design laboratory components that can be produced economically, are very small, and have performance superior to macroscale systems, thus creating a portable and inexpensive gene detection device (15-17). The integration of microelectronic and silicon processing is focused both on the creation of miniaturized chambers, reservoirs, channels, filters, pumps and conduits, in which to carry out cell separation, DNA/RNA extraction and polymerase chain reaction (PCR), and the

creation of unique detection devices or microsensors. Smaller devices demand the use of smaller fluid or tissue samples even getting down to the individual cell level, thus enabling minimally invasive diagnostic procedures. Moreover, smaller amounts of reagents are needed, in terms of tens or hundreds of nanoliters compared with the 100 μLs employed in microtiter plate assays, and reactions in miniature are often more accurate and faster than that on a macro scale. In microelectronic devices, the high surface-to-volume ratio in the hair-breadth channels allows rapid mixing of fluids through diffusion alone, with limited sample fluid evaporation, and the reaction reaches its endpoint much quicker than in a microwell.

Microelectronic technologies can be automated, the most important aspect being integration of all functions in a "Lab-on-a-Chip" configuration (18). In this system the sample is automatically guided from one place to another on the chip until the entire preanalytical (nucleic acid extraction and amplification), analytical (labeling, reaction, and separation) and detection phases are complete, with reduced intrinsic human error and risk of contamination (Figure 1). The ongoing development of novel materials and the application of these innovative processes to molecular biology make possible to set up routine assays on standardized chips allowing the development of high-throughput genotyping.

This review is devoted to the description of the application of microelectronics to all the processes to be faced during a genetic test; in particular, microelectronic applications to sample preparation, DNA amplification and sequence variation detection will be discussed.

MICROELECTRONICS IN PREANALYTICAL STEPS: SAMPLE PREPARATION

The ultimate goal of this technology is miniaturization of the analytical instrumentation to fully integrate multiple processes, including DNA extraction, amplification, hybridization and detection, on a single device. This relies on the application of processes of electronic circuitry combined with micromachining technologies to fabricate sophisticated devices containing a fluidic network of microchannels equipped with pumps, valves, heaters, coolers and filters for mixing, purifying, concentrating, dispensing and separating sample and reagents.

The wide variety of biological specimen and cell types, which need to be processed prior to DNA or RNA analysis, often demands complex preanalytical approaches. Many electric, chemical (19), enzymatic, osmotic, thermal (20) and mechanical (21) methods have been investigated for cell rupture, DNA extraction and purification, which are suitable for integration with high-throughput genotyping platforms (22-24).

In particular, microelectronics can be employed in different stages of sample preparation by exploiting the dielectrophoresis (DEP) methodology (15,25-27). DEP is based on the interactions between the intrinsic dielectric properties of the cells and an applied electric field. By selecting the appropriate frequency of an alternating current electric field, different types of cells can be focused to different regions of the microelectrodes and can be separated for subsequent diagnostic applications. The simplicity and flexibility of this system makes it particularly suitable for chip-based separations of a variety of cells, including bacteria (28-30), cancer cells (31-33),

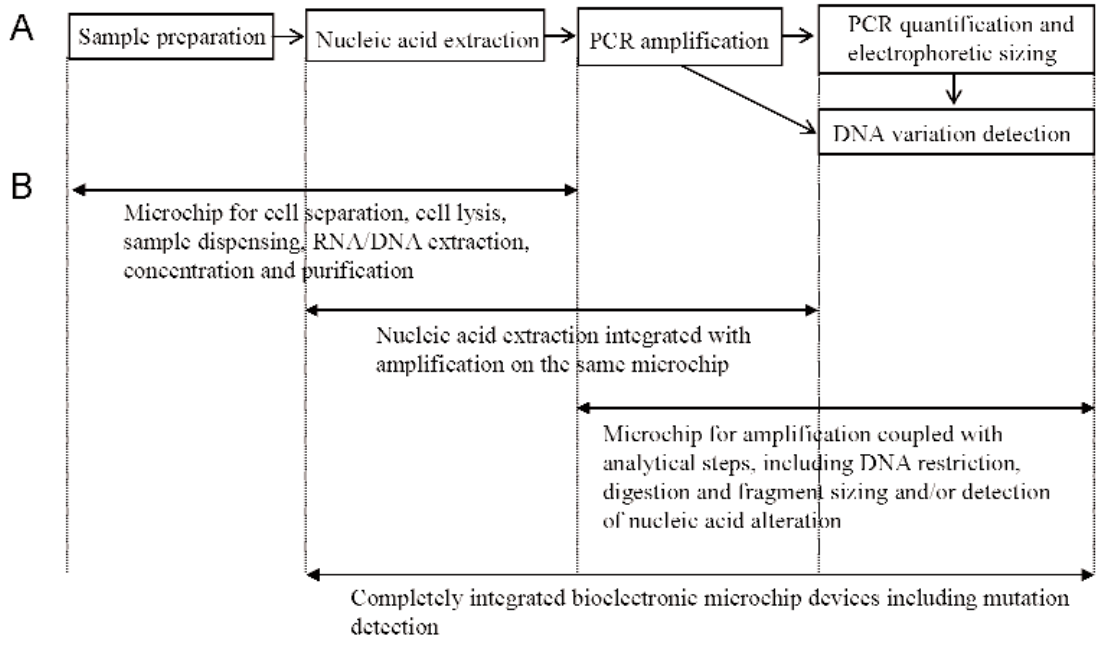

Figure 1
A. Flowchart of a molecular diagnostic procedure; B. Integrated processes where microelectronics can be involved.

stem cells (34), and leukocyte subpopulations (35).

The Cytocon microsystem developed by Evotec is a commercial microsystem for cell separation and sorting through DEP in a Cell Processor Chip that provides handling and analysis of live cells even at the single cell level (32,36). Other systems associate cell isolation through DEP with high voltage pulses which cause cell lysis and DNA release into solution (37,38). Alternative methods for trapping of DNA through DEP have been reported (39,40). Most of these are based on the integration of cell separation and DNA isolation with PCR analysis (see following sections) (27,41,42).

Moreover, Nanogen has developed a system based on microelectronics (43-45). Preparation and hybridization of DNA/RNA from E. Coli is carried out by a two-step process. In a first step, microelectronics are employed to separate E. Coli cells from blood; an alternating current electric field is than used to direct bacteria to microlocations on the chip where they are retained after blood cells have been washed off. Subsequently, the isolated cells are lysed by applying a series of high-voltage pulses to release nucleic acids, including RNA, plasmid DNA and genomic DNA molecules, which are then analyzed on an "assay chip" through microelectronic detection (46).

MICROELECTRONICS IN DNA AMPLIFICATION

A crucial step of most genetic tests is the PCR and often multiplexed amplification formats are needed to analyze a variety of target sequences on the same sample.

PCR is mainly performed through alternating rapid cycles of heating and cooling and a great effort has been spent in miniaturizing this reaction (47-49). Thanks to its thermal properties silicon is extremely well-suited for microfluidic biochip application: its high thermal conductivity (~150W/°C) allows a uniform temperature to be maintained over a wide area of the chip and its low thermal capacity affords fast thermal cycling making the reaction time shorter than that of conventional thermocyclers. Silicon also has the unique feature of allowing electronic controls to monitor the temperature and provide feedback to temperature controller to be integrated on the same piece of silicon. A high surface to volume ratio is advantageous by allowing more efficient thermal transfer and dissipation and resulting into faster cycling times. Indeed, compared to a conventional PCR reaction tube, the reaction chamber of a PCR chip increases 13-fold the surface to volume ratio (50-52). Microfluidic PCR chips are designed to be used once and then discarded because the fluid microchannels are impractical to clean.

Several chips for performing PCR with significantly decreased cycling time and sample volume have been described (4,51,53-57). These include continuous-flow PCR with total reaction times as short as 90 s (Figure 2A) (50). In these systems temperatures can be kept constant over time at different locations and PCR amplification is performed by continuously flowing the sample through three well-defined temperature controlled zones on a glass microchip (4,55). Alternatively, the silicon substrate can heat and cool the fluid sample in place where it remains stationary eliminating the need to transfer it between separate areas of the chip, each at a different temperature, greatly simplifying the chip's microfluid design (Figure 2B).

PCR microchips based on multiple reaction chambers with resistive heaters were also developed (58,59). Among these, microchip devices for cell lysis, multiplex PCR and electrophoretic sizing have been described (60,61). In this systems electrodes placed within the individual reservoirs apply voltages to drive the electrosmotic flow, obviating the need for mechanical pumps or valves, with the channel intersections serving as "virtual injection valves".

Nanogen has explored the possibility to perform the amplification step directly on the chip by coupling microelectronics with Strand Displacement Amplification (SDA) (62). Nanochip technology uses electronics to address biotin-labeled amplification primers to specific position on the array. Consequently, amplification primers are spatially separated still sharing reagents and enzymes. Exploiting this tool, Westin et al. developed assays to simultaneously amplify and discriminate six different gene sequences representative of different bacterial strains allowing multiplex anchored amplification and complex genotype discrimination on the same platform. Moreover, these Authors were able to perform anchored multiplex amplification of 10 different PCR products from both human and bacterial genes (63,64). Nanogen improved this technology by combining amplification and genotyping on the same platform, allowing the detection of Factor V Leiden mutations on human samples (37).

A variety of prototype PCR-chips have been described (52,57,60,65-69). However, generally only those integrating different pre- and post–PCR analytical procedures on the same device have been commercialized so far (48,70).

MICROELECTRONICS APPLIED TO DNA QUALITY CHECK, PCR QUANTIFICATION AND ELECTROPHORETIC SIZING

The area of microfluidics and microfabricated devices has also been successfully exploited to develop high-throughput cost-effective electrically-driven techniques for DNA separation. These include capillary electrophoresis (CE) systems utilizing capillaries etched or formed into glass, silicon, or similar substrates (71-78), free-flow electrophoresis (79,80), micellar electrokinetic capillary chromatography (81), capillary gel electrophoresis (82-85) and sizing of large DNA fragments (78,86,87). A system for amplified DNA quantification by electrochemiluminescence has also been described (88).

Nucleic acid purification is a crucial step in many biological and medical applications. Hong et al. developed microfluidic chips that can sequentially process nanoliter volumes for nucleic acid purification from as little as a single mammalian or bacterial cell (89). In this microde-

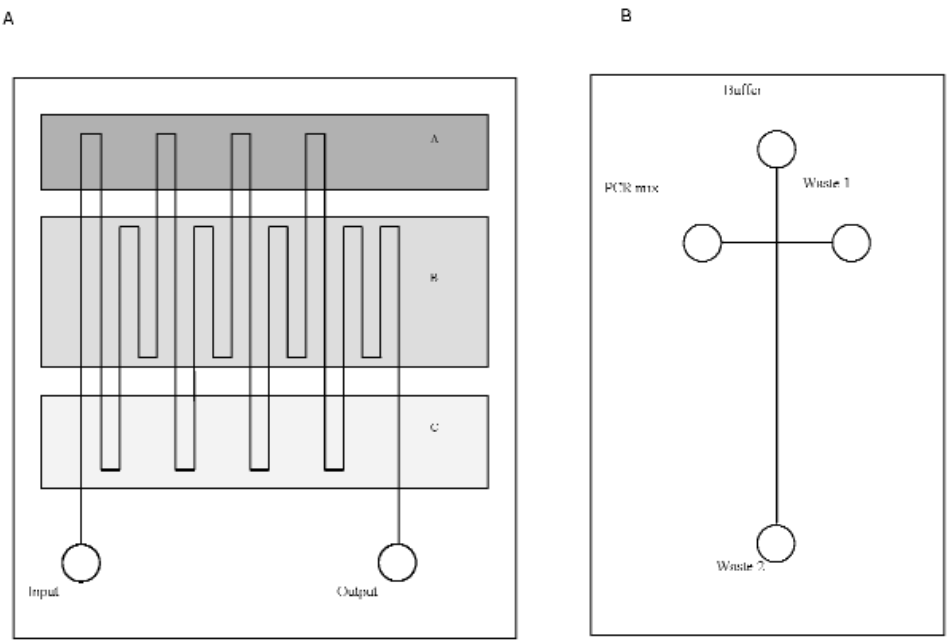

Figure 2
Alternative models of PCR microchip.
A. Continuous-flow PCR microchip: temperature is kept constant at each location (A=annealing, B=extension, C=denaturing temperature) and PCR amplification is performed by continuously flowing the sample through the three temperature controlled zones on the glass microchip (55).
B. Conventional geometry of a microchip based on resistive heaters, equipped with multiple reaction chambers. The fluid sample is heated and cooled in place, being transferred to different areas (58).

vices all processes, such as cell isolation, cell lysis and DNA or mRNA purification, were carried out on different samples in parallel (78,89).

An integrated monolithic microchip for DNA restriction digestion and fragment sizing has also been developed (66). Integration of individual steps including thermal cell lysis to release DNA, simultaneous amplification of multiple gene loci using selected primer sets and electrophoretic analysis of the amplified products on a microchip format has been reported. The devices have been applied to the detection of bacteriophage λ, E. Coli genomic and DNA plasmids (27,60).

A system of CE microchips allowing DNA sizing and proteins separation of samples in parallel with a higher resolution and throughputs than conventional techniques has also been described (90,91). Moreover, commercially available devices for preanalytical and analytical processes by the use of microelectronics have been developed. In particular, a novel microfluidic LabChip technology-based system (Agilent 2100 Bioanalyzer), suitable for separation, sizing, quantifying, and identifying DNA or RNA samples extracted from cells, has been developed by Agilent and Caliper Technologies (92). The disposable LabChip has multiple interconnected reservoirs for samples, sizing ladder, sieving matrix and buffers. These LabChips drive fluid around the chip in photolithographically etched channels by electrokinetic pressure, a combination of electrophoresis and electro-osmosis, in which molecules are charged by their interaction with the capillary wall surfaces (93,94).

If properly designed, these assays can provide quantitative and precise results over a wide dynamic range for detection and quantification of genetically modified organisms, restriction digestion fragment analysis and template quantification prior to cloning or sequencing experiments, and genotyping microsatellites, variable number of tandem repeats and insertion/deletion polymorphisms (95-101). Moreover, a LibraryCard reagent array, i.e. a plastic card 10 cm × 10 cm, has been developed to dry and store nanograms of individual reagents (50). Additionally, more high throughput applications, such as the LabChip 90 and the Caliper 250 system, have been developed. These microfluidics-based electrophoresis methods can be used for analysis of DNA fragments and proteins on a large scale format from 96- or 384-well plates. Electrophoretic separation, fluorescence detection, and electronic data analysis are performed automatically.

A completely different approach was described by Philips Research. This company developed a new technology for driving and controlling fluids into microchannels through electrocapillary pressure (ECP) (http://www.research.philips.com). Based on the fact that the apparent interfacial tension depends on the electrical charge density accumulated at the interface, ECP can be applied for electrically adjusting the magnitude of the capillary effect, and the electrostatic forces on the meniscus can thus be used to control the position and motion of fluids inside a microchannel (93,102). This allows rapid and reversible fluid actuation in three-dimensional microchannel structures in a micrometer scale, at a velo-

city hundred times higher than achieved by nonmechanical and electrical methods.

LabCard devices, exploiting electric fields to move fluids through capillaries on the chip surface for miniaturization, integration, and automation of complex, multistep biochemical processes, were developed by Aclara BioSciences Inc. (50,103). This approach has also been exploited in the electrophoresis-Tag (eTag) assay system, where each specific target (DNA, RNA or protein) in solution is associated with a unique Tag conferring a distinct charge-to-mass ratio and resulting in predefined electrophoretic characteristics. Tens or hundreds samples can be separated and resolved by using CE (85,104).

An alternative strategy for fragment separation, studied by Nakane et al. (105), utilizes nanometer scales pores ("nanopores") as Coulter counters (106) to electronically detect and characterize individual DNA molecules during CE. The reduction in the ion current along an alpha-hemolisin channel due to the presence of individual DNA molecules can be analyzed to provide some information on the nucleotide composition of the DNA molecule (105,107,108). The nanopore is an extremely sensitive detector, yielding an actual count of molecules in its proximity and providing additional information on charge polarity or approximate length, by detecting molecules from tens to thousands of base pairs in length. This approach can discriminate between individual very small secondary structures differing by one nucleotide, providing a powerful tool for sequence-specific DNA detection (85,109,110). The nanopore detector is an electrical system that can be manufactured on the same chip as the separation capillaries, which would be particularly suitable for further miniaturization and incorporation of CE on a chip.

Other systems utilize globular nanoparticle, called nanospheres, associated with a double pressurization technique during microchip electrophoresis for the separation of DNA fragments across a wide range of fragments size (80,111,112).

MICROELECTRONICS IN DETECTION OF DNA VARIATIONS

In the last few years, a number of companies developed completely integrated systems for mutation detection based on the microarray technology (13,48,57,73,113). Among these, highly complex integrated bioelectronic chips that incorporate electronic components are already commercially available. Here we describe in detail principle and instrumentation of some of those which have already been exploited for the development of diagnostic protocols (Nanogen and Motorola) and of some others which seem to be the most promising for the clinical area.

A microarray technology based on the use of microelectronics for the identification of SNPs and mutations in disease-genes was developed by Nanogen (28,43,114-116). The NanoChip Molecular Biology Workstation components include a chip loader, a fluorescent detection/reader, a computer control interface and a data display screen. The NanoChip is a multisite, individually controlled 10 by 10 array of microelectrodes coated with a thin hydrogel permeation layer containing streptavidin. Biotinylated amplicons are automatically placed on the cartridge and electronically addressed to selected pads by positive bias direct current, where they remain embedded through interaction with streptavidin. The DNA at each pad is then hybridized to a mixture of stabilizers and Cy5- and Cy3-labeled oligonucleotide probes, specific for either the wild-type or the mutant sequence. Single base pair mismatched probes are then preferentially denatured with the application of an increasing temperature on the cartridge (Figure 3). After this step of thermal stringency, the array is imaged and the fluorescence quantified. Fluorescence signals emanating from positive test sites are scanned, monitored and quantified by highly developed, digital image processing procedures. A multi-task system controls all the specific aspects of machine operation, including assay execution, fluorescent signal detection, signal processing and data analysis. The fluorescence signal ratios of the reporter probes, discriminating between mutant, heterozygous, and wild-type samples, allow to assess the patient genotype.

The technology can be designed for the detection of many DNA variations located within the same DNA amplicon. Higher density arrays are in development, which have on-chip complementary metal oxide semiconductor (CMOS) control elements that can regulate the currents and voltages to each of the test sites on the array (117). These higher-density devices have on-chip CMOS control elements that can regulate the currents and voltages to each of the test sites on the array. The semiconductor control elements are located in the underlying silicon structure and are not exposed to the aqueous samples that are applied to the chip surface.

Up to now, many application of Nanogen technology for DNA variation identification have been reported in different fields. In particular, test kits are commercially available for mutations detection in the CFTR gene, which is associated with cystic fibrosis; the HFE gene, involved in hereditary hemocromatosis; the Factor V and Prothrombin genes, associated with thrombosis (118-120). This assay has also been applied to a microchip platform integrated with the amplification phase (37), the Apo E (79), aspartoacylase and beta-globin genes (121). Other NanoChip assays have been developed for SNP analysis in different genes (116), such as: MTHFR (119); β2-adrenergic receptor and hepatic lipase (44); cytochines (122); MeCP2 involved in Rett syndrome (123); rearranged during transfection (RET) (124); BRCA1 (125); MEFV (126); paraoxonase 1 (127); N-acetiltransferasi 1 and 2 (128); OGG1 gene, associated with different kinds of human cancers (129); glycoprotein Ia and IIIa, follicle-stimulating hormone receptor, collagene type 6 (130) and retina-specific ABC transporter (ABCA4) (131). Additionally, Nanogen developed the SNPmine technology for the detection of unknown genetic variations. The method is based on enzymatic recognition of mismat-

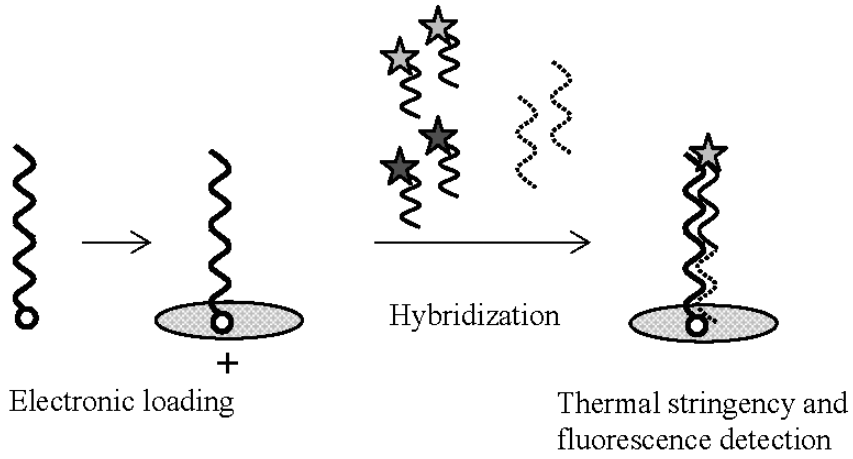

Figure 3
NanoChip assay. Biotinylated amplicons are electronically addressed to the microchip pads by positive current. The DNA at each pad is hybridized to a mixture of stabilizers and Cy5- and Cy3-labeled oligonucleotide probes, specific for either the wild-type or the mutant sequence. After a step of thermal stringency, the mismatched probes are denatured and the fluorescence is quantified.

ched bases in heteroduplexes formed between test DNAs and reference wild-type samples (132).

The Nanogen technology has also been applied to the analysis of simple sequence repeats (133), anchored in-situ amplification (62), expression profiling (79,134), immunoassays and preparation of DNA/RNA from bacteria for pathogen screening (46,83,135).

A platform for electronic detection of nucleic acids on microarrays, the eSensor chip, was introduced by Motorola and successfully applied to mutation detection in disease-genes (81,136,137). This bioelectronic technology relies on the specific generation of electrical current through reversible oxidation and reduction of metal complex labels on nucleic acid targets. The eSensor chips have gold electrodes coated with specific DNA capture probes (25 bases in length) along with reference and auxiliary electrodes. Unlabeled nucleic acid targets are immobilized on the surface of the microchip through sequence-specific hybridization with the capture probe. A separate signaling probe, containing ferrocene-modified nucleotides, is hybridized to the target in the region adjoining the capture probe binding site (Figure 4). When the sandwich complex among the target, capture and signaling probe is perfectly assembled, the ferrocene labels are brought into sufficient proximity to the underlying electrode surface for detection. An alternating current voltage applied to the electrode causes reversible reduction and oxidation of ferrocenes allowing electrons transfer between the label and the electrode surface. The current generated by this system is detected by the electronic detection system called the eSensor 4800, which can analyze 48 chips at a time, each of them containing 16 to 36 electrodes. The single-base mismatch discrimination is achieved through capture or signal probes specifically designed for either the mutant or the wild-type target sequence. Alternatively, the two signal probes are labeled with one of 2 different ferrocene-derivative DNA oligonucleotides characterized by distinct electrochemical potentials thus generating different signals. The detection is performed at the specific temperature at which a complex between a perfectly matched target is significantly favored compared to a mismatched target, which is inhibited. eSensor chips have been used for different clinical applications, e.g. genotyping chips for hereditary hemochromatosis and cystic fibrosis (137,138). A chip for gene expression monitoring has also been developed that surveys five genes differentially regulated in the cellular apoptosis response (137).

PharmaSeq Inc. applies microtransponder technology to detect and differentiate large numbers of unique DNA sequences in a single assay. Each microtransponder is an integrated circuit composed of photocells, electronic memory, clock and antenna. DNA capture probes covalently linked to the microchip surface recognize a specific complementary target DNA sequence tagged with a fluorescent dye (Figure 5). The microtransponder electronic memory stores information to identify the sequence of the oligonucleotide probe attached to its surface. For the assay, the transponder is placed in a solution containing the fluorescently labeled DNA target and then flows through a capillary tube and pasts a laser beam. The laser light induces fluorescence of dye molecules on the surface of the microtransponder (positive result), while microchips not coated with labeled target DNA will only transmit an identification signal (negative result). At the same time the laser light activates the microtransponder, which transmits a radio-frequency identification signal. A scanner, i.e. a high-speed flow fluorometer modified to detect radio frequency, detects both the fluorescent signal generated by the labeled nucleic acid hybridized to the probe on the microtransponder and the identification number of the microtransponder. When an assay is performed with many probes (and thus, many microtransponders), the scanner identifies the microtransponders involved in the reaction, thus

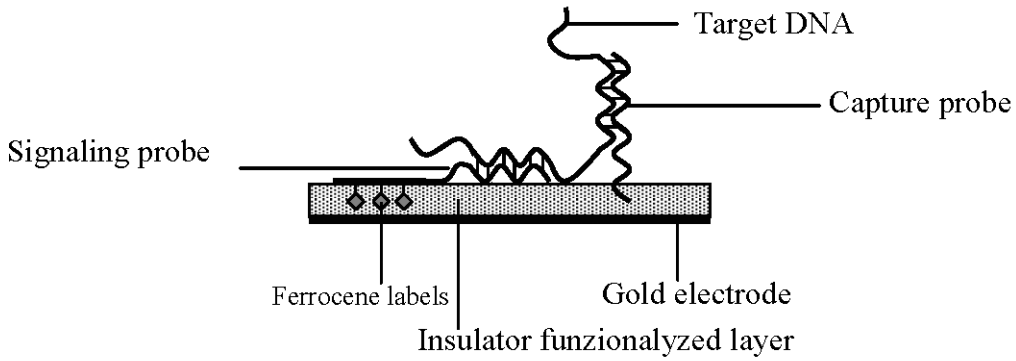

Figure 4
eSensor microchip. The system is based on gold electrode functionalized with the DNA probe sequence. The target sequence, after being captured onto the electrode by the capture probe, hybridizes also to a second reporter sequence (signal probe), labeled with ferrocene. The target is not labeled but is 'sandwiched' by the capture probe and signal probe. The interfacial electron transfer from ferrocene to the gold electrode is then detected.

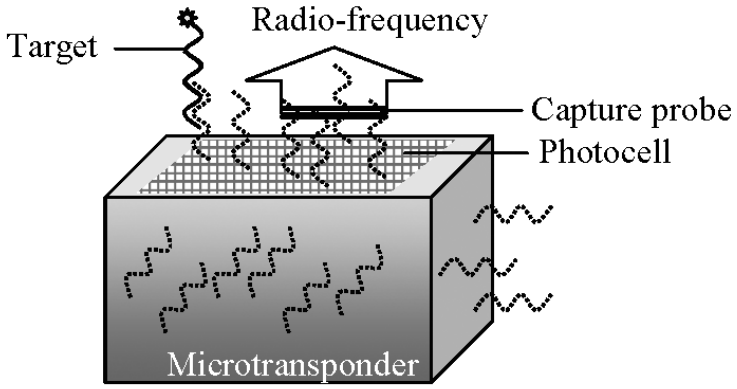

Figure 5
PharmaSeq microtransponder-based assay. Thousand copies of capture probes are bound to each microtransponder and recognize only specific target fluorescent-labeled DNA, which is different from one microtransponder to another. A laser light scans both the fluorescent signals and the radio frequency of the microtransponder, thus identifying the sequence of the attached capture probe and analyzing the hybridization event.

identifying positive DNA probe hybridizations. Thousand probe copies are attached to each transponder, different transponders bear different probes, and a small aliquot of liquid may contain 10,000 transponders specific for genetic diseases or mutations (139,140). Protein detection assays have also been performed successfully. The DNA microchip family of microtransponders has been recently enriched by the last generation of nanotransponders measuring just 250 x 250 x 100 µm.

STMicroelectronics is developing a silicon chip for DNA analysis that integrates both DNA amplification and detection (http://www.st.com). The prototypes is a disposable device which performs the PCR in 12 microscopic channels buried in the silicon each measuring 150×200 µm. Electrical heating elements (resistors) in the silicon heat the channels, cycling the mixture at precise temperatures for DNA amplification. The system achieves temperature control by an external electronic system, which drives the on-chip heaters by following real-time the temperature value, which is read by integrated sensors, and continuously monitoring and adjusting the parameters of the reaction. The reaction channels are located at a few µm to the sensing elements thus allowing the readings to closely follow the actual temperatures seen by the samples. When the sample has been sufficiently amplified, the system fluidically moves the amplified DNA into the biochip's detection area, where gold electrodes are preloaded with DNA probes. Irreversible DNA grafting on electrodes is performed by using electrochemical polymerization of pyrrole-modified nucleic acids: by applying a voltage to the gold coated electrode, the pyrrole groups copolymerize to form polypyrrole, which then sticks to the electrode (a technology developed in collaboration with LETI/French Atomic Energy Commission -CEA-, Grenoble, France) (141-143). PCR amplification is carried out in the presence of a biotin-labeled nucleotide precursor. Detection of hybridization is performed by the use of phycoerytrin-streptavidin markers and subsequent fluorescence detection following laser excitation. The platform comprises a disposable cartridge, a bench-

top-sized control instrument and a reader that manage the interface between the operator and the biochip (59).

Today, the Cepheid technology is the only commercially available system providing a fully-integrated platform for the detection of microbial species from different starting biological samples based on real time PCR (144,145), even if it cannot be completely considered on a microscale. Cepheid developed the GeneXpert system which combines cartridge-based sample preparation with amplification and signal detection in an integrated, automated DNA analysis instrument (146-148). The platform has a modular design to be integrated into a wide range of system configurations for rapid, automatic DNA-based analyses to purify, concentrate, detect, and identify targeted DNA sequences in less than 30 min. The DNA may come from a complex sample such as blood, biological tissues, food, or soil. Cell separation and concentration are carried out with modular filter and solid-phase holder assemblies; cell lysis is performed by ultrasonic techniques and DNA or RNA capture, enrichment and purification are carried out by chips or solid-phase materials (54,149). This instrument uses silicon-based reaction chambers with integrated electronics heaters and sensors to control the reaction temperatures. Four-color detection within the module enables real-time identification and quantification of four different targets in the same reaction tube.

Several research groups developed completely different approaches for a possible application in molecular diagnostics by the use of microelectronic microchips. Among these, a silicon-based thermal-gradient microarray for DNA genotyping with independently temperature-controlled test sites has been developed by Fortina et al. (150). In this system, each of the 20 or 100 separately controllable hybridization sites contains a heater and a temperature sensor on a silicon dioxide/nitride surface. The surface of the thermally controlled regions, named “islands”, is previously treated with different reactive groups and allele-specific oligonucleotide probes are subsequently attached to discrete spots on the chip within the islands. Hybridization is then performed with Cy5-tagged targets PCR. These thermal gradient DNA chips were tested for four different sequence variations in factor VII and hemochromatosis genes (150).

Another system, the electrochemical DNA (E-DNA) sensor, utilizes electrodes linked to molecular beacon-like DNA stem–loop with a redoxable label. Hybridization between the target DNA and the complementary sequence at the hairping induces a conformational change in the DNA hairping structure, which alters the distance between the electrode and the electroactive reporter, leading to signal variation at the electrodes (94,151,152).

A biosensor based on magnetoresistance technology, the Bead ARray Counter (BARC), was developed by Edelstein et al (153,154). This instrument can measure, even at the level of single molecules, the forces that bind DNA-DNA complementary strands. Hundreds of microfabricated magnetoresistive sensors are fabricated on a chip and DNA probes are patterned on it. The target sample is labelled with magnetic beads and hybridizes with the complementary probes on the microarray. Beads on the surface are detected by sensors as change in magnetoresistance, which is proportional to the number of beads on the surface. Thus, signal intensity and signal location represent concentration and identity of the target. The BARC biosensor has been applied to the detection of biological warfare agents (153).

In the last few years a higher level of miniaturization of material and technologies is being also applied to molecular diagnostics. Nanotechnology-based chips have been produced on a nanometer-scale for nanomanipulation: nanoscale sensing elements such as carbon nanotubes and semiconducting nanowires have been incorporated in electronic biosensors and the detection is at the level of atoms, molecules or supramolecules structures (155-157) The reduced scale of the assay to nanoscale allows quick selection of substances and more sensitive and flexible analysis (158,159). Several technologies are based on nanoparticle, like magnetic nanobeads and gold nanoparticle as described in the following section, and this approach will probably reach the diagnostic market within the next few years.

DNA BIOSENSORS

DNA biosensors are electrochemical devices that have received a special attention in the last few years and represent a fascinating application of microelectronics to SNP genotyping and molecular diagnostics.

Molecular-based biosensors utilize highly specific biological reactions to detect target analytes combined with a signal transducer that translates the biorecognition event into a usable electronic readout signal (160-163). Due to the robust and specific base-pairing interactions between complementary sequences, DNA is especially suitable for biosensing applications in molecular diagnostics and novel designs for DNA-based electrochemical sensing have recently appeared. These are based on platforms containing single-stranded probe sequences immobilized within a recognition layer, which recruit the target DNA to the surface and facilitate formation of the probe-target complex, combined with electrochemical transducers in such a way that the binding event triggers a useful electrical signal. The hybridization event is commonly detected via variation in the current signal at the transducer or other hybridization-induced changes in electrochemical parameters (e.g. conductivity or capacitance). These systems have the advantage to potentially obviate the need for PCR amplification of the target and should facilitate genetic readouts from single cells and even single molecules, further accelerating the realization of large-scale genetic testing.

A variety of different biosensors relying on a wide range of sensitive electrochemical signaling strategies have been described. Here they are classified into two main categories, based on the absence or presence of a covalently linked label indicator. The first category includes systems based on a label-free detection approach, where the hybridization event can be detected thanks to the specific intrinsic electroactivity of DNA yielding redox

signals due to base reduction or oxidation or to deoxyribose oxidation. The second category includes systems employing an indicator-based detection, where redox-active indicator molecules bind specifically to the DNA probe-target complex.

Label-free electrochemical detection

Although label-dependent methods achieve the highest sensitivities (164-166), avoiding the labeling steps can simplify the readout and increase the speed and ease of nucleic acid assays, with the advantage of monitoring hybridization in real time. Detection can be performed with an inexpensive analyzer allowing to develop portable formats for clinical testing (167,168).

The strategy firstly described for DNA sensing was based on reduction and oxidation of DNA at an electrode, where the amount of DNA reduced or oxidized reflected the amount of DNA captured (160). The label-free electrochemical detection can be accomplished by monitoring changes in the intrinsic electroactivity of the nucleic acid target or probe or changes in the electrochemical properties of the interface. Among the four DNA bases, guanine is most suitable for label-free electrochemical detection since it is most easily oxidized. The use of the electrocatalytic action of a $Ru(bpy)_3^{3+}$ redox mediator greatly amplifies the guanine oxidation peak resulting from the hybridization event, thus creating a catalytic cycle that results in a increase of the current output (Figure 6) (169,170). This approach has been exploited to detect base substitutions or deletions/insertions involving guanine bases. Several different applications have been reported, such as the X-fragile myotonic distropy, factor V Leiden, cystic fibrosis, BRCA1 breast cancer gene and cytochrome P450 3A4 (171-176). It has also been shown that the catalytic currents due to guanine oxidation resulting from the hybridization event increases with the number of repeats in a linear function of the number of repeats in the immobilized DNA strand (174,177). The hybridization signal gets more stringent using peptide nucleic acid (PNA) probes (172).

Alternatively, cathodic stripping measurements of the target adenine for sensitive detection of DNA hybridization has been reported (173,178,179).

In order to overcome the problem of the high background current signal, methodologies based on DNA probes immobilized onto magnetic beads have been developed. Following hybridization of captured target sequences, the beads are magnetically separated from the analytes and the guanine/adenine oxidation/reduction is detected (154,173,180,181).

A different approach to identify DNA sequence variations is based on detecting a probe-target hybridization with the measurement of different rates of electron transfer through single stranded (ss)- and double stranded (ds)-DNA. When a mutation is present, the current flow is altered by a change in base stacking at the mismatched site, perturbating charge migration through DNA (170,182). In this case hybridization can be detected from the redox response of an electroactive marker that associated with the double strand DNA molecule (162). Useful redox-active markers include metal chelates such as $Co(phen)_3^{3+}$ was used (183), bisbenzimide dyes such as Hoechst 33258 (184,185), anthracycline antibiotics such as daunomycin (186,187) or ferrocenyl naphthalene diimide (188). In particular, $Co(phen)_3^{3+}$ was used for detecting the cystic fibrosis ΔF508 deletion sequence and a daunomycin-based biosensor was applied to the study of Apo E polymorphisms. An application on a chip based-format was demonstrated by Boon et al. (183,189). Detection of point mutation on a gold electrode modified with thiolated DNA was accomplished by monitoring changes in a DNA-mediated charge transport between an electroactive methylene blue intercalator and a ferricyanide redox species. In this assay, reduced methylene blue reduces ferricyanide in solution, thus causing additional electrons to flow to methylene blue. If

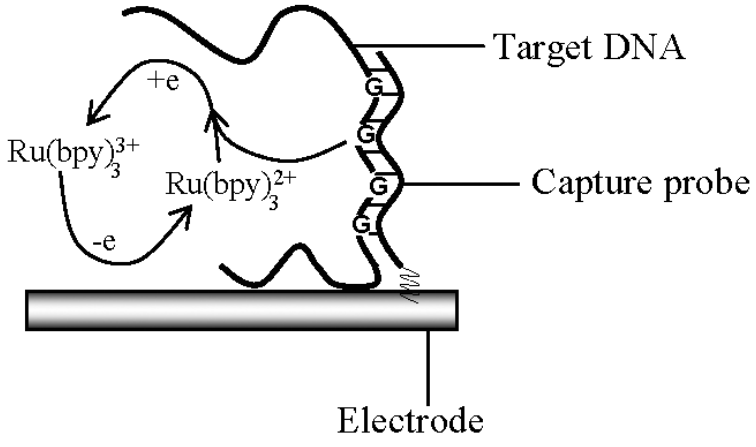

Figure 6
Label-free electrochemical detection. Hybridization of target DNA is detected by monitoring changes in the intrinsic electroactivity of nucleic acids. In this example the electric signal is amplified by using the electrocatalytic action of a $Ru(bpy)_3^{3+}$ redox mediator, which is reduced by guanine oxidation and then is oxidized by the electrode, thus regenerating the red-ox reaction.

the DNA contains a mismatch, the bound methylene blue is not catalytically active and the electrochemical signal is greatly diminished. By this approach, any single-base mismatch could be detected (189). The same approach with a redox-active marker was also coupled with an oligonucleotide enrichment at plastic electrodes through an amplification step and subsequent electrohybridization (172).

A different approach to direct, label-free, electrical detection of DNA hybridization has also been performed by monitoring changes in the conductivity of conducting polymer molecular interfaces, e.g. using DNA-substituted or doped polypyrrole films (Figure 7). It has been demonstrated that a biosensor using an electroactive polypyrrole functionalized with an oligonucleotide film displays a decreased current response during the duplex formation, caused by bulky conformational changes along the polymer backbone due to its higher rigidity subsequent to the hybridization event (166,190,191).

In an alternative approach, a self-assembled bilayer lipid membranes was used (192,193). The formation of a double stranded DNA molecule causes a decrease in the ion conductivity across the lipid membrane surface, allowing the direct monitoring of DNA hybridization. This application was implemented by the use of indirect redox markers and PNA probes (194).

A different label-free method was developed by Fritz et al., who created a field-effect sensor based on an electrolyte-insulator-silicon structure (195). Variations in the insulator-electrolyte surface potential arose from the binding of nucleic acids to the insulator surface modifying the charge distribution in the silicon below the electrolyte (195).

Indicator-based electrochemical detection

In contrast to the label-free based approach, where markers intercalate the double stranded-DNA following the hybridization event, several strategies have been applied in which target DNA sequences or reporter probes are covalently labeled with electroactive indicators such as ferrocene derivatives and osmium tetroxide complexes or enzymes taking part to electrocatalytic processes (187,196,197). Alternatively, a three-component "sandwich" assay has been described, in which the redox labeled oligonucleotide has a tag matching a synthetic sequence specifically designed to bind a protruding portion of the probe-target complex, eliminating the need to modify the target strand (see Figure 4 as an example) (160,198). This dual-hybridization approach has been coupled, for example, with ferrocene tags by Motorola for the above mentioned eSensor DNA microchip (199,200). Incorporation of a second redoxactive marker (dimetylcarbammmyl ferrocene) with a different activation potential allows the simultaneous detection of two targets without spatial separation (201,202).

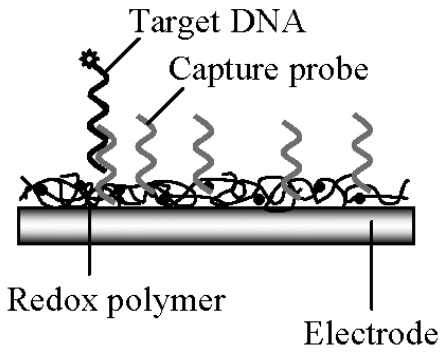

Figure 7
Label-free electrochemical detection on redox polymer. Capture probes are linked to an electron-conducting polymer interfaces. The formation of a duplex molecule following the hybridization event changes the conductivity of the membrane surface, caused by a variation in the ion permeation.

Colloidal gold nanoparticles have also been introduced in a variety of sandwich-based assays (203). In particular, one study reported the electrochemical detection of the Factor V Leiden trough probe strands immobilized on a pencil graphite electrode (204), and a similar procedure was also reported for the detection of human cytomegalovirus, for a noncompetitive heterogeneous immunoassay for immunoglobulin, and for other biomedical applications (205-207).

A new electrochemical coding bioassay exploiting nanoparticle labels with different redox potentials to encode DNA sequences has been developed (208,209). The technique is based on the use of different inorganic-colloid (quantum dots) nanocrystal tracers, whose metal components yield highly sensitive stripping voltammetric signature with distinct electrical hybridization signals for the corresponding DNA targets. Probe-modified magnetic beads are hybridized with target DNA, magnetically separated from the pool of analytes and hybridized again with the nanoparticle-labeled reporter strands. The resulting products are isolated, and the nanoparticles are dissolved and analyzed. Simultaneous electrochemical detection of three targets was demonstrated with cadmium sulfide, zinc sulfide and lead sulfide nanoparticle tags in connection with a sandwich hybridization assay and stripping voltammetry of the corresponding heavy metals (Figure 8).

The sandwich-based approach has also been coupled with an enzymatic reaction to generate or amplify electrical signals for electrochemical detection of DNA hybridization. In this method the enzyme can be directly conjugated with the probe or can be associated with avidin to recognize the biotin-labeled probe. The enzymatic reaction catalyzes the precipitation of an insoluble product on the electrodes or on a piezoelectric quartz crystal providing the detection of the hybridization/DNA recognition event (206,210-212).

Patolsky et al. (211) described the use of an oligonucleotide probe labeled with alkaline phosphatase which allows the oxidative hydrolysis of 5-bromi-4-chloro-3-indolyl phosphate to an insoluble derivative leading to the formation of a negatively charged insulating film on the electrode. This precipitation changes the conductivity of the system also allowing the quantitative determina-

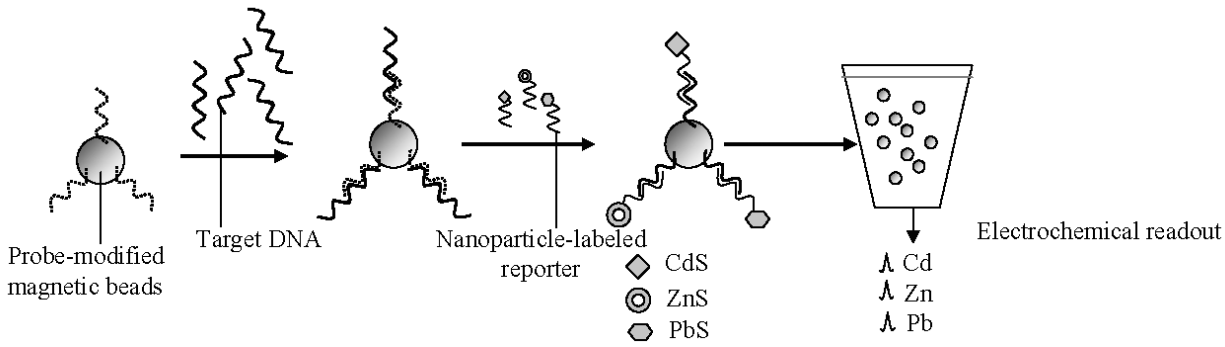

Figure 8
Electrochemical coding bioassay with nanoparticle labels. Magnetic beads are modified with three probes specific for three different target sequences. Beads are then magnetically separated from analytes and hybridized with reporters labeled with three nanoparticles encoding for distinct electrical hybridization signals indicating the corresponding DNA targets [zinc sulfide (ZnS), cadmium sulfide (CdS) and lead sulfide (PbS)]. The resulting products are isolated and the nanoparticles are dissolved and analyzed, allowing the simultaneous electrochemical detection of the three targets.

tion of the target DNA (Figure 9). In an alternative approach, the alkaline phosphatase is conjugated with avidin to recognize a biotin-labeled probe (213).

The enzymatic precipitation has been also associated with a single nucleotide extension protocol to detect a mutation causing the Tay-Sachs genetic disorder. In this approach the base complementary to the mutant target is labeled with biotin. Its interaction with the avidin/alkaline phosphatase conjugate catalyzes the precipitation of an insoluble product on the electrode by changing the conductivity at the support. This analytical procedure has been applied to mutation detection directly on genome DNA without PCR amplification (212,214).

Alternatively, horseradish-peroxidase, soybean peroxidase, or β-galactosidase label targets were used (181,213). Alkaline phosphatase and β-galactosidase were together used to differentiate two DNA targets by measuring the chronopotentiometry of their electroactive products allowing amplified dual-target electrochemical detection (215). Oligonucleotide-functionalized liposomes or biotin-labeled liposomes binding to the oligonucleotide-DNA assembly are negatively charged and, when deposed on the electrodes, alter their interfacial properties, thus enabling the electrochemical transduction of the amplified sensing processes. Biotin-labeled liposome can associate in a dendritic-type structure, greatly amplifying the signal. This approach was applied to the detection nucleic acid sequences (213).

DNA biosensors described in the previous sessions have been used mainly on experiments with synthetic DNA targets of about the same length as the oligonucleotide capture probes on the microchip surface. Much lower sensitivity and specificity are reached when the assay is performed on real, substantially longer target DNA molecules such as PCR products, plasmid, viral or chromosomal DNAs. The main difficulties arise from considerable interactions of redox indicators with non specific DNA or with the single-stranded hybridization probe (196). To overcome this problem, a new strategy based on the physical separation of DNA hybridization from electrochemical detection has been recently developed. Particulary, the hybridization of target DNA with the immobilized DNA probe is performed at one surface (called surface H) while electrochemical detection is carried out at another surface (detection electrode) (180). By this approach, subnanomolar concentration of target DNA can be determined and long DNA target molecules can be analyzed. Moreover, a wider variety of detection electrodes can be exploited, which are easier to integrate into microfluidic systems. The two-surface system allows much easier setting up of optimal hybridization and detection conditions than the one-surface systems. For example, Palecek et al. and coworkers used commercially available magnetic Dynabeads oligo(dT)25 as surface H, in connection with various detection techniques, including both label-free and indicator-based electrochemical approaches (180,216). Enhanced amplification of DNA sensing processes was also achieved by using tagged liposome as probes for the amplification of DNA sensing events (217,218).

A different signal amplification strategy can be obtained with nanoparticles (77,203,219). A system with small arrays of microelectrodes with gaps between the electrodes is constructed. Capture probes are immobilized on the substrate between electrodes. A sandwich hybridization between target and detector probe, conjugated to gold nanoparticles, close the microelectrode gap (Figure 10): hence, DNA hybridization creates an electrical circuit for the detection of SNPs. The detection limit for target DNA is in the range of 500-fmol (5×10^{-13} mol), so the method has the required sensitivity for DNA detection without a PCR step, at least for certain applications.

CONCLUSIONS

There are around 10,000 single gene disorders, most

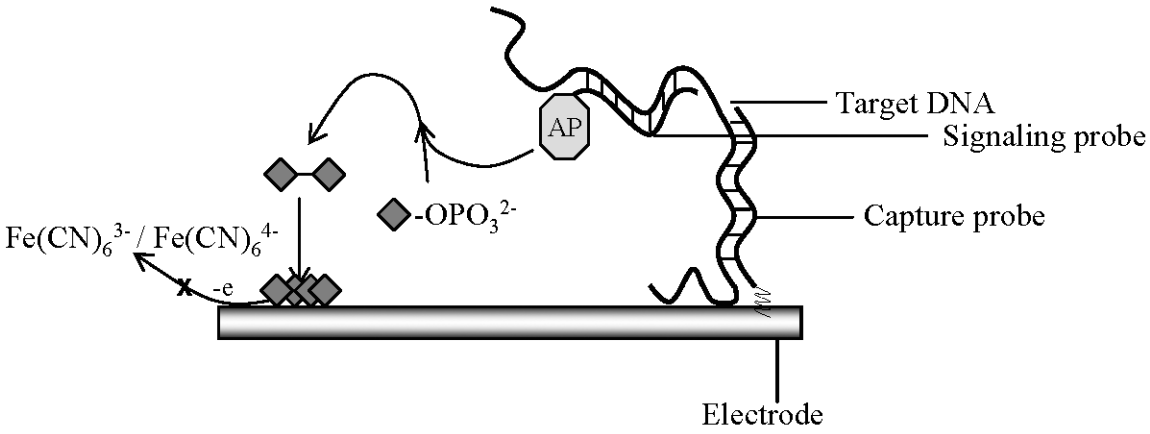

Figure 9
Indicator-based electrochemical detection. The example describes the enzymatic amplification of DNA hybridization signal, where an enzyme takes part to electrocatalytic processes. The labeling enzyme is alkaline phosphatase (AP), which is covalently bound to the 5'-end of a signaling probe specific for the target molecule. The association of AP with the sensing interface biocatalyzes the precipitation of an insoluble dimer (♦ -♦) product from 5-bromo-4-chloro-3-indolyl phosphate (♦ -OPO_3^{2-}), providing the amplification of the hybridization signal, which is detected as an increased resistance to electron transfer between the electrode and ferricyanide in solution.

are rare, but together they affect up to 5% of the population. Genetic tests are commercially available for about 200 of such disorders, but more are urgently needed. Genetic factors also underline many common diseases. A substantial research effort is required to investigate the genetic basis of these diseases and to develop new methods of analysis. Predisposition and pharmacogenetic tests are likely to require screening for small variations in DNA sequence (SNPs). These are simple to test for, but there will be millions of these possible genetic variations, often needing to be tested in combination in a large number of individuals. In the incoming years, high-throughput testing technology will be required to vastly increase speed and ability to do more tests and to deliver quality results quickly and more economically.

Over the past few years, we have witnessed a tremendous progress towards the development of microfabricated devices for DNA analysis. In particular, many technological advances have become possible through the application of microelectronics. Electronic techniques are of particular interest for this purpose because they can be directly integrated with microelectronics and microfluidics systems to gain advantages in miniaturization, multiplexing, and automation. The combination of different microelectronic applications allow the integration of sample collection, DNA extraction, amplification, hybridization and signal detection into miniaturized portable "lab-on-a-chip" systems performing the tasks of several large instruments. These systems are very powerful tools that researchers can use to better understand the molecular bases of genetic diseases and complex traits. They can also be useful diagnostic tools able to provide optimal, economical and technical, conditions, for the screening of inherited diseases in the general population.

Although to date the microarray technology has been applied mainly in research settings, it is developing so rapidly that soon it will probably find its place in the clinical laboratory.

Up to now only a few of these completely integrated systems allow to get a final genetic result directly starting from a blood sample. Moreover, only a few systems for high-throughput automated SNP/mutation detection

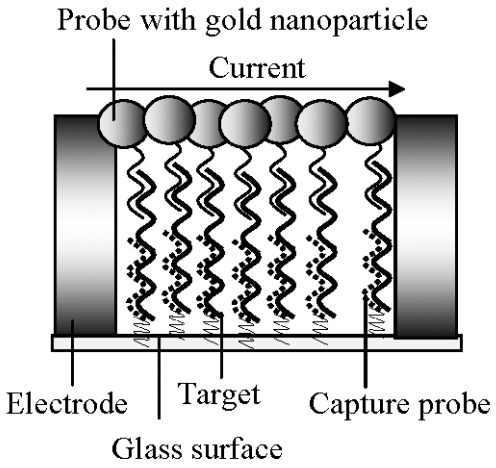

Figure 10
Nanoparticle probes for signal amplification. The three-component sandwich assay is carried out on capture probes immobilized on the substrate between two electrodes. Targets and detector probes conjugated to gold nanoparticles hybridize to capture probes creating an electrical circuit which closes the gap between microelectrodes, which is detected as a decrease in the resistance of the system. The very high sensitivity of the approach allows detection of single-base mutations within the target sequence.

and some others are presently commercially available, even if several diagnostic assays have been developed for large-scale SNP/mutation detection of widespread genetic diseases. Nevertheless, many novel promising approaches have been described which might be exploited to produce ready-to-use instrumentation. The future trend is to exploit the enormous opportunities offered by DNA-based sensors. These represent innovative routes to interface at the molecular level the DNA-recognition and signal transduction elements allowing to monitor the actual hybridization event with high sensitivity and speed. The final goal of all these integrated microfabricated microelectronic chips will be to provide important new tools for molecular diagnosis of genetic diseases.

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